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The Patent Office

Cardiff Road
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1. Your reference	REP05981GB	
2. Patent application number *(The Patent Office will fill in this part)*	19 APR 1999	9908932.8
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	Cambridge University Technical Services Ltd The Old Schools Trinity Lane Cambridge CB2 1TS United Kingdom	
Patents ADP number *(if you know it)*		6916809004
If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom **See continuation sheet for further applicant(s)**	
4. Title of the invention	MICROSCOPY	
5. Name of your agent *(if you have one)*	GILL JENNINGS & EVERY	
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Broadgate House 7 Eldon Street London EC2M 7LH	
Patents ADP number *(if you know it)*	745002	

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77 Continuation Sheet

Our reference: REP05981GB

Applicant Details

Imperial College Innovations Limited
47 Princes Gate
London
SW7 2QA
United Kingdom

Country/State of incorporation: United Kingdom

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	1
Description	8
Claim(s)	1
Abstract	
Drawing(s)	1 +1

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. For the Applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date
19 April 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward
0171 377 1377

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MICROSCOPY

The cell is the most fundamental unit of living organisms, whether animal, plant or micro-organism. Thus, studying the structure of cells, their composition and how their various constituents function would lend valuable insight in understanding the complex processes that occur in biological systems. This requires techniques that allow investigation of cell samples to be conducted in real-time, non-invasively and in a liquid that mimics physiological conditions so that their functionality is retained. Although satisfaction of these criteria is non-trivial, the emergence of scanning probe microscopy (SPM) has made it possible to study samples under these conditions.

In SPM, when a sharp probe tip is scanned in close proximity with respect to the sample under study, interactions between the tip and the sample come into play as a result of the chemical/physical properties of the sample. Thus, plotting the tip-sample interactions as a function of the tip's position with respect to the sample allows generation of a profile of the property that gave rise to the measured interaction in the first place. Members of the SPM family that are commonly applied for biological imaging are atomic force microscopy (AFM) (Henderson et al., 1992; Radmacher et al., 1992; Schoenenberger et al., 1994; Lal et al., 1995; Ohnesorge et al., 1997), scanning ion-conductance microscopy (SICM) (Proksch et al., 1996; Korchev et al., 1997) and scanning near-field optical microscopy (SNOM) (Mertesdorf et al., 1997; Keller et al., 1998; Subramaniam et al., 1998).

In AFM, the interaction forces between the tip and sample are measured to obtain the topographic features of the sample; it can be operated in contact or non-contact modes. Contact AFM, particularly the "tapping in liquid" mode of operation (Putman et al., 1994) has been used to image living cells with a resolution that is one order of magnitude better than conventional optical microscopy. However, interpretation of the obtained results has been difficult since the nature of the interaction forces that come into play between the tip and the sample are not fully understood as is the extent of deformation/perturbation of the "soft" cell membrane structure by the hard AFM tip.

SICM and SNOM differ in the mechanisms implemented for distance regulation between the tip and sample as well as in the nature of the scanning probes that are used. In SICM, an electrolyte-filled, glass micropipette is scanned over the surface of an insulating sample, which in turn is bathed in an electrolytic solution. The pipette-sample separation is maintained at a constant value by monitoring the ion-current that flows via the pipette aperture, between two electrodes placed inside the pipette and electrolyte solution respectively. For an applied bias between the electrodes, the ion-current signal depends on a combination of the micropipette's resistance, R_P, and the access resistance, R_{AC}, which is the resistance along the convergent paths from the bath to the micropipette opening. R_P depends on the tip diameter and cone angle of the micropipette whereas R_{AC} displays complicated dependence on the sample's electrochemical properties, geometry and separation from the probe. It is R_{AC} that lends ion-current sensitivity to the pipette-

sample separation and allows its exploitation in maintaining the two at distances such that contact does not occur. The tip's output is used to generate topographic features and/or images of the local ion-currents flowing through pores on the sample surface. The spatial resolution achievable using SICM is dependent on the size of the tip aperture. Although fabrication of small apertures (<100nm) is possible (Brown and Flaming, 1986) these tips prove to be fragile and break when scanning samples that are not flat. Probes that have allowed SICM to be established as a non-contact profiling method of elaborated surfaces have tip-diameters that typically range between 100nm and 1.5μm and give rise to corresponding values of resolution (Hansma et al. 1989; Proksch et al., 1996, Prater et al., 1991).

In SNOM, light is coupled down a fibre-optic probe with an output aperture of sub-wavelength dimensions which is scanned above the sample surface. Interaction forces between the tip and sample are used to maintain their separation at a value that roughly corresponds to the sub-wavelength dimensions of the aperture. This arrangement allows simultaneous generation of optical and topographic images whose resolution depends on the size of the output aperture and the tip-sample separation. Whilst it is straightforward to fabricate probes with smaller apertures (Hoffmann et al., 1995; Sayah et al., 1998), achieving smaller tip-sample separations in liquid (<60nm) is difficult because of the occurrence of tip-sample crashes which rupture the near-field probe and damage the

sample. The best reported resolution to date of SNOM operated in liquid is 60nm (Keller et al., 1998).

From the brief review above, it is evident that although SICM and SNOM are not capable of AFM resolution, they achieve values that are comparable to each other. Thus, the two techniques are well-matched and their respective benefits (SICM as a non-contact profiling method and SNOM as a technique that allows acquisition of optical information pertaining to a sample) could be used to advantage if they were implemented in one experimental arrangement.

In this paper, we report modification of an existing SICM experiment such that simultaneous generation of SICM and SNOM images of living cardiac myocyte cells was made possible. Cardiac myocyte cells were chosen for study for two main reasons. Firstly, they are composed of light and dark bands of material / striations that periodically occur every 2.5μm which give them a distinct appearance and therefore, make them a good model system for study. Second and most importantly, they constitute the muscle that lines the heart's chambers where their involuntary, synchronous contraction is crucial to the pumping activity of blood in the heart (Sommer and Jennings, 1991).

The following illustrates the invention.

The SICM experiment consists of components that feature in all SPMS, namely, scanning probe, piezo-actuator scanning elements, control electronics and a computer. These components are built in and around an inverted microscope (Diaphot 200; Nikon Corporation, Tokyo, Japan) central to the experiment.

We fabricate our SICM probes by pulling borosilicate, glass microcapillaries with outer and inner diameters of 1.00mm and 0.58mm respectively, using a laser-based micropipette puller (Model P-2000, Sutter Instrument Co., San Rafael, CA, USA). Probes with conical taper lengths and apex diameters of 200nm, 400nm and 1.0μm respectively were made reproducibly and easily in this fashion.

Three-dimensional and high precision movement of the probe relative to the sample is achieved by the piezo-translation stage (Tritor 100, Piezosystem Jena, Germany) on which the SICM probe is mounted. The stage has a range of 100μm in the x,y,z directions so that scanning over biological samples with features that scale up to 30-50μm is made possible. The high voltages required for deformation of the piezo-ceramic material that facilitate the stage's movement are provided by high voltage amplifiers (System Enviso, Piezosystem Jena, Germany). These amplifiers respond to appropriate signals generated by the control electronics to drive the piezo-translation stage and achieve movement of the tip relative to the sample. In addition to being connected with the hardware aspect of the microscope, the control electronics is interfaced with a computer that allows data

acquisition and image analysis. The control/data acquisition hardware and software are produced by East Coast Scientific (Cambridge, U.K).

The pipette-sample separation is maintained at a constant value by monitoring the ion-current that flows between Ag/AgCl electrodes in the micropipette and electrolyte solution which the sample is immersed in. For this work, Phosphate Buffer Saline (PBS) solution is used for both filling the micropipette and the electrophysiological medium of the cardiac myocyte cells so that concentration cell potentials and liquid junction potentials are not established. The ion-current is measured for DC voltages of 50-200mV applied to the electrodes. It is amplified by means of a high-impedance operational amplifier (OPA129, Bull Brown International, USA) and converted to a voltage signal over a resistance of $10^9\Omega$. This signal is then input into the control electronics where it is used for feedback control and data acquisition.

The micropipette is housed in a special, custom-made holder which is assembled together with the current amplifier and piezo-translation stage to comprise the SICM head. The SICM head is mounted onto the arm of the inverted microscope's z-translator which facilitates coarse vertical positioning of the micropipette relative to the sample. positioned immediately below it. The sample is contained in a petri-dish which is placed on the microscope's stage. Movement of the sample relative to the micropipette is achieved by the x,y translation controls of the stage. The processes of monitoring the

vertical position of the micropipette relative to the sample and selection of an area of interest on the sample can be viewed on a TV screen via a video camera (JVC TK-1280E, Victor Company, Japan). The video camera is also connected to a "frame-grabber" equipped with database and archive management facilities (Acquitas IDA, Dynamic Data Links Ltd., Cambridge, UK) which are used for coordinating optical and scanned images.

Modifications were made to the experiment described above in order to permit simultaneous SICM and SNOM imaging. Laser light (Laser 2000 UK Ltd) of wavelength, 532nm, was coupled via a multi-mode fibre (FG-200-UCR; 3M Specialty Optical Fibers, West Haven, USA) into the micropipette. In order to ensure confinement of light to the aperture, 100-150nm of aluminium was evaporated onto the walls of the pipette.

A micropeptide of the type that may be used in this invention is illustrated in the accompanying drawing.

CLAIMS

1. Apparatus for imaging an object, comprising a probe via which light may be delivered; a sensor to detect ion current; and means for controlling the position of the probe relative to the object in response to the ion current.

2. Apparatus according to claim 1, wherein the probe is a micropipette.

3. Apparatus according to claim 1 or claim 2, wherein the outer surface of the probe is coated, e.g. with a metal layer, to prevent leakage of light.

4. Apparatus according to any preceding claim, wherein the probe comprises a fibre optic.

5. Apparatus according to any preceding claim, which comprises a laser light source.

6. A method for imaging an object in a liquid environment, by scanning ion-conductance microscopy, using a probe whose distance from the object is maintained in response to the ion current in the liquid, wherein the probe includes means for delivering light to the object.

7. A method according to claim 6, wherein the distance is less than the wavelength of the light.

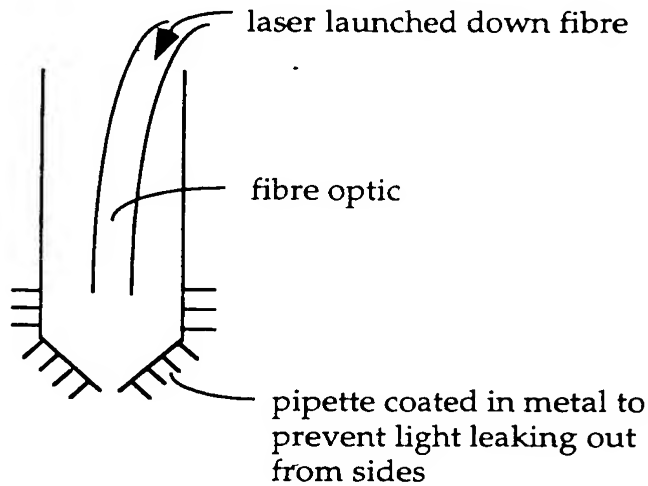
laser launched down fibre
fibre optic
pipette coated in metal to
prevent light leaking out
from sides

FIGURE 1